REGULATOR OF NOTCH SIGNALING ACTIVITY

The present invention relates to a novel regulator protein and to nucleic acid that encodes this protein. The invention also relates to methods of diagnosis and therapy of disease using this protein and nucleic acids encoding therefor.

The Notch signalling pathway comprises an intracellular signalling mechanism that is essential for proper embryonic development. This pathway was first identified and studied in Drosophila, although comparative analyses have since demonstrated a remarkable degree of functional conservation between many of these developmentally important genes in Drosophila and their vertebrate counterparts. The Notch locus has been shown to comprise an evolutionarily conserved cell interaction mechanism that plays a fundamental role in controlling the progression of immature cells to a more differentiated state.

The *Notch* genes encode transmembrane receptors that help to determine cell fate during development. Notch acts as a receptor in a signalling pathway that when activated, may block or delay the progression of immature cells toward a more committed state. Inappropriate activation or inactivation of Notch can lead cells to adopt an incorrect fate.

The mechanism of signalling downstream of Notch receptors remains uncertain. However, it is thought that a proteolytic cleavage releases the cytoplasmic portion of Notch which then translocates to the nucleus and participates in the specific activation of genes whose products inhibit differentiation.

Mutations in *Notch* genes are linked to various diseases including some cancers (Zagouras *et al*, 1995; Capobianco *et al*, 1997; Gallahan and Callahan, 1997; Gridley, 1997). Indeed, two members of the mammalian Notch gene family were initially identified as oncogenes and at least three distinct members of the Notch gene family (Notch 1 , Notch 2 and Notch 4) can contribute to neoplasia in mammals. It seems that truncation of human Notch 1 plays a causal role in the formation of T cell neoplasms. Furthermore, an association has been shown between mutations in the Notch3 gene and the CADASIL phenotype (cerebral autosomal dominant artieriopathy with subcortical

infarcts and leukoencephalopathy). Notch function may also be linked to neurodegenerative disease by virtue of its connection to Presenilin (Levitan and Greenwald, 1995; de Strooper *et al*, 1998). This assertion is based on the study of genetic interactions in *C. elegans* in which a putative functional link between Notch and presenilin gene products has been noted. Presenilins are linked to processing of amyloid precursors, suggesting a potential role in the genesis of Alzheimer's disease.

However, the demonstration of a link between a particular disease and a specified genotype does not directly facilitate the development of a therapeutic strategy to treat or prevent the disease. This is particularly true for those linkages set out above, since although ligands of Notch have been identified, the signalling cascade downstream of the receptors remains incompletely understood.

It is likely that genes encoding proteins that regulate Notch signalling or that participate in Notch signalling may also be linked to various disease states. Identification of genes that modify Notch activity may thus provide the basis for a nucleic acid or protein diagnostic or therapeutic tool.

There is thus a need for the identification of molecules that affect the levels of activity of Notch and that might act as suitable targets for therapeutic agents for treatment of diseases linked to mutations in the Notch locus. Such proteins would also be useful in identifying drugs that modify Notch activity.

Summary of the invention

According to a first aspect of the present invention there is provided a protein comprising an amino acid sequence as identified in SEQ. ID. No. 1, or a functional equivalent thereof.

This novel protein, termed Notchless, was identified in a screen for dominant modifiers of a Notch mutant phenotype in the Drosophila wing. The mutant dominantly suppresses the wing notching phenotype of *notchoid* mutations and the Notchless protein is shown herein to bind to the cytoplasmic domain of Notch. Notchless modifies Notch signalling activity in a variety of Notch-dependent signalling processes in both *Drosophila* and *Xenopus* embryos.

By the term "functional equivalent" is meant any compound that possesses the same conformation as a domain of the Notchless protein that is responsible for its physiological function. Accordingly, this term is meant to include any macromolecule or molecular entity that mimics the conformation of the Notchless protein or that possesses an equivalent complementarity of shape to that possessed by the binding sites of the Notchless protein whose sequence is identified in SEQ ID 1.

Included in the invention as functional equivalents are invertebrate and vertebrate homologues of the Drosophila Notchless protein, with the proviso that the protein is not the Xenopus protein, the sequence of which is available on the accession number AF069737.

Also available on the genome database is a mouse EST (AA396500), a human EST (AA341327) and an *S. cerevisiae* DNA sequence (1351791). The *C. elegans* sequence was compiled by the inventors from multiple clones (C48486, D70156, C35601, M89091) and has a gap in the 6th WD40 repeat (this sequence was not identified as a gene by the *C. elegans* genome project). Until now, no function has been ascribed to any of these nucleic acid sequences in regulating Notch.

The term "functional equivalents" is also intended to include fragments or variants of the Notchless protein or closely related proteins exhibiting significant sequence homology. By "fragments" is meant any portion of the entire protein sequence that retains a physiological function of the wild type Notchless protein, such as for example, an ability to bind specifically to Notch. Accordingly, fragments containing single or multiple amino acid deletions from either terminus of the protein or from internal stretches of the primary amino acid sequence form one aspect of the present invention. "Variants" include mutants containing amino acid substitutions, insertions or deletions from the wild type Notchless sequence.

The Notchless protein is thought to function by binding to the cytoplasmic domain of Notch. It is thought (although the applicant does not wish to be limited by this theory) that Notchless also binds to protein factors other than Notch, since mutants that reduce or remove Notchless expression have been shown to increase Notch activity (see Examples).

The predicted Notchless protein has a novel highly conserved N-terminal domain followed by 9 WD40 repeats (Figure 6A). The WD40 repeat is found in a wide variety of proteins of diverse function and is thought to be a protein interaction domain (reviewed in Neer *et al.*, 1994). Typically, WD40 proteins contain 7 repeats. Structure analysis of β-transducin suggests that these form a propeller-like structure and that 7 repeats can pack to make a flat cylinder (Neer and Smith, 1996).

Notchless is unusual in that it appears to contain 9 WD40 repeats. Repeats 5 and 6, though recognisable as WD motifs, appear quite divergent in that they lack particular signature residues of the WD40 repeat (not shown).

Blast searches using the amino-terminal sequence (before the first WD repeat) identified closely related sequences identified in yeast, *C. elegans*, man and mouse. In all cases the N-terminal domain is followed by WD repeats. In the human and mouse, only short EST fragments have been sequenced; until now, no function has been assigned to these sequences.

Degenerate PCR using primers directed against conserved sequences in the N-terminal domain of the mouse and human proteins was used to isolate a *Xenopus Nle* cDNA. The *Xenopus* protein also contains 9 WD repeats with strong similarity to the *Drosophila* and *C. elegans* proteins. We note that particular WD40 repeats are more similar between species than they are to other WD40 repeats in the protein of the same species. Together this suggests that these proteins represent true orthologues. Database searches suggest that there may only be one member of this gene family in *C. elegans*, mouse and the human.

Sequence comparison indicates that the degree of conservation in the N-terminal domain is quite high among the different family members (Figure 6B). In the 80 amino acid region corresponding to residues 27-106 of Notchless, sequence identity ranges from 33% between *Drosophila* and *S. cerevisiae* to 61% between *Drosophila* and *Xenopus* proteins. Particular residues are identical in all species examined, suggesting that they may be important for domain structure. This domain has been termed the Nle domain.

Orthologues of the Notchless protein are thus thought to retain their function across different species and to effectively regulate the activity of a protein member of the Notch gene family from a different species. By gene family is meant a group of genes whose products share a common function and that exhibit common sequence homology. By common sequence homology is meant that the gene sequences are related by divergence from a common ancestor. The protein products of the genes may share common motifs in their sequence.

According to a further aspect of the present invention there is provided a Notchless protein or functional equivalent thereof modified at one or more positions in the sequence of the protein by the substitution, insertion or deletion of one or more amino acids from the wild type sequence shown in SEQ ID No. 1. Furthermore, such proteins may contain single or multiple amino acid deletions from either terminus of the protein or from internal stretches of the primary amino acid sequence.

The Notchless protein or functional equivalent thereof may be prepared by any suitable means as will be clear to the man of skill in the art. Preferably, the protein is generated by recombinant DNA technology by expression of the encoding DNA in an expression vector in a host cell. Such expression methods are well known to those of skill in the art and many are described in detail in *DNA cloning: a practical approach, Volume II: Expression systems*, edited by D.M. Glover (IRL Press, 1995) or in *DNA cloning: a practical approach, Volume IV: Mammalian systems*, edited by D.M. Glover (IRL Press, 1995). Protein compounds may also be prepared using the known techniques of genetic engineering such as site-directed or random mutagenesis as described, for example, in *Molecular Cloning: a Laboratory Manual*: 2nd edition, (Sambrook *et al.*, 1989, Cold Spring Harbor Laboratory Press) or in *Protein Engineering: A practical approach* (edited by A.R. Rees *et al.*, IRL Press 1993).

For many applications, a protein according to the present invention may be fused to an effector or reporter molecule such as a label, toxin or bioactive molecule. Such molecules may comprise an additional protein or polypeptide fused to the Notchless protein, or functional equivalent, at its amino- or carboxy-terminus or added internally. The purpose of the additional polypeptide may be to aid detection, expression, separation or purification of the protein or may be to lend additional properties to the protein as desired.

In particular, it is envisaged that fusion proteins comprising Notchless, or a functional equivalent thereof may be used as a "platform" to deliver biologically active protein domains to Notch. For example, an enzyme could be fused to Notchless or to a functional equivalent, that could be targeted to Notch in this fashion. Such a fusion partner would preferably be regulated in some fashion so that it would only be activated when the Notch and Notchless proteins physically interact. This may be through some conformational change, so that only when the Notch binding domain of Notchless interacts with the Notch protein is the fusion partner activated. Another mechanism may be through the inclusion in the fusion protein of a regulatable domain such as a hormone regulation domain, so that the activity of the fusion partner may be limited until the relevant drug is provided.

A particular enzyme of choice as a fusion partner might be a protease. Such an enzyme would act to "clip" the activated form of Notch that is expressed in some cancers, so inactivating it. As will be clear to those of skill in the art, any effector protein (or effector domain) could be used that is capable of modifying Notch activity *in vivo*. Post-translational modifications, such as phosphorylation (where kinase or phosphatase is the effector enzyme), or ubiquitination would also have the desired effect.

Other suitable candidates for fusion will be reporter molecules such as luciferase, green fluorescent protein, or horse radish peroxidase. Labels of choice may be radiolabels or molecules that are detectable spectroscopically, for example fluorescent or phosphorescent chemical groups. Linker molecules such as streptavidin or biotin may also be used. Additionally, other peptides or polypeptides may be fused to a Notchless protein. Suitable peptides may be, for example, β-galactosidase, glutathione-S-transferase, luciferase, polyhistidine tags, secretion signal peptides, the Fc region of an antibody, the FLAG peptide, cellulose binding domains, calmodulin and the maltose binding protein. Antibodies or peptides used to target the Notchless protein more efficiently towards a site of action (for example antibodies against membrane proteins of mast cells) may also be fused to the Notchless protein.

These fusion molecules may be fused chemically, using methods such as chemical cross-linking. Suitable methods will be well known to those of skill in the art and may comprise for example, cross-linking of the thiol groups of cysteine residues or cross-linking using

formaldehydes. Chemical cross-linking will in most instances be used to fuse non-protein compounds, such as cyclic peptides and labels.

When it is desired to fuse two protein molecules, the method of choice will often be to fuse the molecules genetically. In order to generate a recombinant fusion protein, the genes or gene portions that encode the proteins or protein fragments of interest are engineered so as to form one contiguous gene arranged so that the codons of the two gene sequences are transcribed in frame.

According to a further aspect of the invention there is provided a process for the identification of an agent capable of modifying the levels of expression or activity of a Notch protein comprising screening a Notchless mutant in a sensitised Notch genetic background with a drug and selecting for an altered phenotype.

Preferably, the Notchless mutant is an insect larva. For example, compound screening may be performed by feeding larvae of a suitable genetic background on food containing the compound of interest. Modified function is then assessed in the adult fly. This allows the identification of compounds suitable for oral delivery that would either enhance or suppress the severity of the mutant phenotype through either increasing or decreasing the activity of Nle with respect to Notch.

One advantage of such a screen is that it makes possible the selection of drugs that modify Notch activity. Furthermore, these drugs will be suitable for oral delivery. According to a further aspect of the present invention there is provided a drug identified by such a screen.

According to a further aspect of the present invention there is provided a nucleic acid sequence comprising:

a) the sequence of SEQ ID No 2, or

b) a sequence that encodes on expression the amino acid sequence encoded by the sequence of part a), or

c) a fragment of the DNA sequence of part a) or part b) that comprises a nucleotide sequence that encodes the binding domain of Notch.

The preferred nucleic acid molecule, according to the invention, comprises a nucleotide fragment identical to or complementary to any portion of the nucleotide sequence shown in SEQ ID No 2, that encodes a Notchless protein. It will be appreciated that individual or multiple nucleotide insertions, deletions and substitutions may be made without departing from this aspect of the invention.

The nucleic acid molecule may comprise DNA, cDNA or RNA. Preferably, the nucleic acid molecule comprises DNA.

According to a further aspect of the invention there is provided a probe capable of screening for Notchless and prepared from the DNA sequence of SEQ ID No 2. The probe preferably comprises at least 15 oligonucleotides, more preferably between 15 and 300 oligonucleotides, most preferably between 15 and 50 oligonucleotides.

The invention also includes cloning and expression vectors containing the DNA sequences of the invention. Such expression vectors will incorporate the appropriate transcriptional and translational control sequences, for example enhancer elements, promoter–operator regions, termination stop sequences, mRNA stability sequences, start and stop codons or ribosomal binding sites, linked in frame with the nucleic acid molecules of the invention.

Additionally, in the absence of a naturally-effective signal peptide in the protein sequence, it may be convenient to cause the recombinant protein to be secreted from certain hosts. Accordingly, further components of such vectors may include nucleic acid sequences encoding secretion signalling and processing sequences.

Vectors according to the invention include plasmids and viruses (including both bacteriophage and eukaryotic viruses). Many such vectors and expression systems are well known and documented in the art. Particularly suitable viral vectors include baculovirus-, adenovirus- and vaccinia virus-based vectors.

The expression of heterologous polypeptides and polypeptide fragments in prokaryotic cells such as *E. coli* is well established in the art; see for example *Molecular Cloning: a Laboratory Manual*: 2nd edition, Sambrook *et al.*, 1989, Cold Spring Harbor Laboratory Press or *DNA cloning: a practical approach, Volume II: Expression*

systems, edited by D.M. Glover (IRL Press, 1995). Expression in eukaryotic cells in culture is also an option available to those skilled in the art for the production of heterologous proteins; see for example O'Reilly *et al.*, (1994) *Baculovirus expression vectors - a laboratory manual* (Oxford University Press) or *DNA cloning: a practical approach, Volume IV: Mammalian systems*, edited by D.M. Glover (IRL Press, 1995).

Suitable vectors can be chosen or constructed for expression of Notchless proteins, containing the appropriate regulatory sequences, including promoter sequences, terminator sequences, polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate. Vectors may be plasmids, viral e.g. bacteriophage, or phagemid, as appropriate. For further details see *Molecular Cloning: a Laboratory Manual*. Many known techniques and protocols for manipulation of nucleic acid, for example, in the preparation of nucleic acid constructs, mutagenesis, sequencing, introduction of DNA into cells and gene expression, and analysis of proteins, are described in detail in *Short Protocols in Molecular Biology*, Second Edition, Ausubel *et al.* eds., (John Wiley & Sons, 1992) or *Protein Engineering: A practical approach* (edited by A.R. Rees *et al.*, IRL Press 1993). For example, in eukaryotic cells, the vectors of choice are virus-based.

A further aspect of the present invention provides a host cell containing a nucleic acid encoding a Notchless protein or functional equivalent. A still further aspect provides a method comprising introducing such nucleic acid into a host cell or organism. Introduction of nucleic acid may employ any available technique. In eukaryotic cells, suitable techniques may include calcium phosphate transfection, DEAE-Dextran, electroporation, liposome-mediated transfection or transduction using retrovirus or other viruses, such as vaccinia or, for insect cells, baculovirus. In bacterial cells, suitable techniques may include calcium chloride transformation, electroporation or transfection using bacteriophage.

Introduction of the nucleic acid may be followed by causing or allowing expression from the nucleic acid, e.g. by culturing host cells under conditions for allowing expression of the gene.

In one embodiment, the nucleic acid of the invention is integrated into the genome (e.g. chromosome) of the host cell. Integration may be promoted by inclusion of sequences which promote recombination with the genome, in accordance with standard techniques.

Transgenic animals transformed so as to express or overexpress in the germ line one or more Notchless proteins or functional equivalents as described herein form a still further aspect of the invention, along with methods for their production. Many techniques now exist to introduce transgenes into the embryo or germ line of an organism, such as for example, illustrated in Watson *et al.*, (1994) Recombinant DNA (2nd edition), Scientific American Books.

According to a further aspect of the present invention there is provided a method of gene therapy of a pathological condition caused by a gene mutation in a patient comprising administering to a patient a nucleic acid encoding a Notchless protein or functional equivalent, in a therapeutically-effective amount.

Suitable diseases include various cancers such as human acute lymphoblastic leukaemia (Capobianco et al., 1997), cervical squamous carcinoma and adenocarcinoma (Zygouros et al., 1995), and mammary tumours (Gallahan & Callahan, 1997; Gridley, 1997) and certain neurodegenerative diseases, for example familial Alzheimer's disease.

The nucleic acid may be introduced into a patient by any suitable means, as will be clear to those of skill in the art. Effective methods of introduction include the use of adenovirus, adeno-associated virus, herpes virus, alpha virus, pox virus and other virus vectors that serve as delivery vehicles for expression of the gene. See generally, Jolly (1994) *Cancer Gene Therapy* 1:51-64; Kimura (1994) *Human Gene Therapy* 5:845-852; Connelly (1995) *Human Gene Therapy* 6:185-193; and Kaplitt (1994) *Nature Genetics* 6:148-153. Retroviral vectors may also be used (see Tumor Viruses, Second Edition, Cold Spring Harbor Laboratory, 1985.) Preferred retroviruses for the construction of retroviral gene therapy vectors include Avian Leukosis Virus, Bovine Leukaemia, Virus, Murine Leukaemia Virus, Mink-Cell Focus-Inducing Virus, Murine Sarcoma Virus, Reticuloendotheliosis Virus and Rous Sarcoma Virus.

The term "therapeutically effective amount" as used herein refers to an amount of a therapeutic agent to treat, ameliorate, or prevent the disease or condition, or to exhibit a

detectable therapeutic or preventative effect. The precise effective amount for a subject for a given situation can be determined by routine experimentation and is within the judgement of the clinician. An effective dose will typically be from about 0.01 mg/ kg to 50 mg/kg or 0.05 mg/kg to about 10 mg/kg of nucleic acid construct.

Non-viral strategies for gene therapy also exist that utilise agents capable of condensing nucleic acid molecules, delivering these molecules to cells and protecting them from degradation inside the cell. Vehicles for delivery of gene therapy constructs may be administered either locally or systemically.

Such strategies include, for example, nucleic acid expression vectors, polycationic condensed DNA linked or unlinked to killed adenovirus alone (see Curiel (1992) *Hum Gene Ther* 3:147-154) and ligand linked DNA (see Wu (1989) *J Biol Chem* 264:16985-16987). Naked DNA may also be employed, optionally using biodegradable latex beads to increase uptake. Other methods will be known to those of skill in the art.

Liposomes can act as gene delivery vehicles encapsulating nucleic acid comprising a gene cloned under the control of a variety of tissue-specific or ubiquitously-active promoters. Mechanical delivery systems such as the approach described in Woffendin *et al* (1994) *Proc. Natl. Acad. Sci. USA* 91(24):11581-11585 may also be used.

Direct delivery of gene therapy compositions will generally be accomplished, in either a single dose or multiple dose regime, by injection, either subcutaneously, intraperitoneally, intravenously or intramuscularly or delivered to the interstitial space of a tissue. The compositions may also be administered directly into a tumour or lesion. Other modes of administration include oral and pulmonary administration, using suppositories, and transdermal applications, needles, and gene guns or hyposprays.

According to a further aspect of the invention there is provided a pharmaceutical composition comprising a Notchless protein or functional equivalent according to the first aspect of the invention, in conjunction with a pharmaceutically-acceptable excipient. Suitable excipients will be well known to those of skill in the art and may, for example, comprise a phosphate-buffered saline (0.01M phosphate salts, 0.138M NaCl, 0.0027M KCl, pH7.4), a liquid such as water, saline, glycerol and ethanol, optionally also containing mineral acid salts such as hydrochlorides, hydrobromides, phosphates,

sulphates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. Auxiliary substances such as wetting or emulsifying agents, pH buffering substances, may also be present. A thorough discussion of pharmaceutically acceptable excipients is available in Remington's Pharmaceutical Sciences (Mack Pub. Co., N.J. 1991).

A carrier may also be used that does not itself induce the production of antibodies harmful to the individual receiving the composition, and which may be administered without undue toxicity. Suitable carriers are typically large, slowly metabolised macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, and inactive virus particles. Pharmaceutical compositions may also contain additional preservatives to ensure a long shelf life in storage.

According to a yet further aspect, the present invention provides a method of treatment of cancer or of a neurodegenerative disease in a patient comprising administering to a patient a Notchless protein or functional equivalent in a therapeutically-effective amount.

According to a yet further aspect, the present invention provides for the use of a Notchless protein or functional equivalent, a nucleic acid encoding a Notchless protein or functional equivalent or of a pharmaceutical composition containing a Notchless protein or functional equivalent in therapy.

According to a still further aspect of the invention there is provided the use of a Notchless protein or functional equivalent according to the invention in conjunction with a pharmaceutically-acceptable carrier in the manufacture of a medicament for the treatment or prevention of cancer or of a neurodegenerative disease in a human or an animal.

The invention also comprises the use of components according to the invention in a diagnostic kit for the detection of mutations present in the genome of a patient. Preferably, such a component will comprise a Notchless protein or functional equivalent, a nucleic acid encoding a Notchless protein or functional equivalent or an antibody that specifically recognises a Notchless protein or functional equivalent. Other desirable components for inclusion in such a kit will be clear to those of skill in the art.

All documents mentioned in the text are incorporated herein by reference.

Various aspects and embodiments of the present invention will now be described in more detail by way of example, with particular reference to the Notchless gene isolated from Drosophila. It will be appreciated that modification of detail may be made without departing from the scope of the invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Comparison of sequences of Notchless proteins from *Drosophila*, yeast, *C. elegans*, mouse, human and *X. laevis*.

Figure 2: Genetic interactions between *Notchless* and *notchoid*.

(A) Cuticle preparation of a wild-type wing.

(B) Wingless protein expression in a wild-type wing imaginal disc visualized by antibody staining. The arrow indicates the stripe of Wingless at the DV boundary.

(C) Cuticle preparation of a nd^1 wing (genotype nd^1 /Y; note that *Notch* is on the X-chromosome so males carry only one copy of the gene).

(D) nd^1/Y; $Nle^{k13714}/+$ wing. The notching of the wing is completely suppressed.

(E) nd^1/Y; $Su(H)^{AR9}/+$. Removing one copy of the *Su(H)* gene enhances the severity of the notching of the wing. Wingless expression in a disc of the same genotype is shown at right.

(F) nd^1/Y; $Su(H)^{AR9}/Nle^{k13714}$ wing. Removing one copy of *Nle* suppresses the notching of the nd^1/Y; *Su(H)*/+ wing and enhances the loss of veins. Wingless expression is restored to normal.

(G) nd^{fa}/Y wing.

(H) nd^{fa} /Y; $Nle^{k13714}/+$ wing. The notching of the wing margin is completely suppressed. Veins are normal in this genotype.

Figure 3: Cloning the *Notchless* gene.

(A) schematic representation of the *Nle* locus.

(B) *Notchless* phenotype (suppressed nd^1 phenotype) produced when one copy of *Nle* is mutated in a nd^1 fly.

(C) Wing from a fly of the genotype as in panel B which also carried a UAS-Nle transgene on the second chromosome. Placing the 1.5 Kb transcript under C765-GAL4 regulation restores the nd^1 phenotype (arrow).

Figure 4: *Notchless* enhances *Abruptex* mutant phenotypes

(A) Wild-type wing, thorax and head cuticles. Veins 1-5 are numbered. The red arrows in the central panel indicate two of the large bristles on the thorax. The blue shading in the right panel indicates the cluster of three orbital bristles above the eye.

(B) $Abruptex^{28}$ mutant wing, thorax and head cuticles.

(C) $Abruptex^{28}$ $Nle^{\Delta 8}/+$ mutant wing thorax and head cuticles.

Figure 5: Genetic interactions between *deltex* and *Notchless*

(A) $deltex^1$ mutant wing.

(B) $deltex^1$ $Nle^{\Delta 8}/+$ mutant wing.

(C) Heat-shock Deltex overexpression under mild conditions produces no phenotype in an otherwise wild-type wing.

(D) Comparable heat-shock Deltex treatment causes loss of veins in a $Nle^{\Delta 8}/+$ wing (arrow).

Figure 6: Molecular features of Notchless protein

(A) Schematic representation of Notchless protein and its orthologues. The conserved Nle domain is indicated in dark gray. WD40 repeats are numbered 1-9 (white numbers).

Percent identity to the *Drosophila* protein are indicated for the Nle domain and for individual WD40 repeats.

(B) Comparison of Nle domains. Sequence identity is highlighted in black, similarity in grey. Similarities are not highlighted if shared by fewer than four proteins. Dashes indicate gaps introduced to accommodate extra residues in the yeast protein. +15 aa indicates a larger insertion.

Figure 7: Expression of *Xenopus Notchless* during embryonic development and phenotypic effects of Notchless overexpression on formation of primary neurons.

(A) Temporal expression of *XNle*.

(B) Spatial expression of *XNle*.

(C) Phenotypic consequence of overexpression of *XNle*, *DNle* and an activated form of *Xenopus NotchI* (XN-ICD) on primary neurogenesis.

Figure 8: Notchless binds Notch *in vitro*

(A) Binding of Nle, Numb-N and Numb-C to GST-Notch-ICD and GST control proteins.

(B) Immunoprecipitation of Notch and Nle expressed in S2 cells.

EXAMPLES

Materials and methods

Drosophila strains

l(2)k13714 is from the BDGP P-element lethal collection. P-element excisions were generated by providing a chromosomal source of transposase activity. 105 w^- excision lines were isolated. One of these was not able to suppress the nd^1 phenotype and was therefore reverted to wild type. Others were analysed for imprecise excision of the P-element by Southern blots. $Su(H)^{SF8}$ and $Su(H)^{AR9}$ are described in Schweisguth and Posakony, 1992. Ax^{28} is described by de Celis and Garcia-Bellido, 1994. $deltex^1$ and pCaSpeR hs-dx are described by Diederich, *et al.*, 1994; Matsuno *et al.*, 1995. nd^1, nd^2,

nd fa and Dp(1;2)51b are described in Lindsley and Zimm (1992) "The genome of Drosophila melanogaster", *Academic Press, San Diego.* w^{1118} was used as wild-type control for cuticle preparations. For heat shock experiments, *pCaSpeR hs-dx/+* and *pCaSpeR hs-dx/Nle* pupae were heat shocked twice for 1 hour at 37°C between 0 and 24 hours after pupariation.

Antibodies

Mouse monoclonal anti-Wg is described in Brook and Cohen, 1996. Mouse monoclonal anti-Notch C17.9C6 is described in Fehon *et al.*, 1990. Mouse (12CA5) anti-HA and rabbit (HA-11) anti-HA were obtained form BabCo.

Constructs for rescue and expression

A 15Kb *Sal*I genomic fragment of phage Y2-6 was inserted into the *Xho*I site of the transformation vector pCaSpeR4. UAS-*Nle* was prepared by cloning the 1.5Kb *Nle* cDNA as a *Not*I-*Xho*I fragment into pUAST (Brand and Perrimon, 1993). An HA-tagged version of Nle was generated by introducing three copies of the HA epitope (YPYDVPDYA) immediately downstream of the first Methionine residue. The *Bam*HI-*Asc*I fragment of pKS-Nle was replaced by a corresponding PCR fragment amplified using the following primers:

5' CGGATCCAAA AAATGTATCC CTATGACGTC CCCGATTATG CCTACCCTTA CGATGTACCT GACTACGCGT ATCCGTACGA CGTTCCGGAC TATGCTCAGG AGACGGACA CGGAGCAAGA GGCCACGCCA CATACGATAC AGGCGCGCCA A 3' and

5' TAAACGAGGC GCGCCTATCG TAT 3'.

pMT-HA-*Nle* was generated by cloning HA-*Nle* as a *Bam*HI-*Sal*I fragment into the inducible expression vector pRmHa-3. pRmHa-3-*Notch* is described in Fehon *et al.*, 1990.

GST-fusion protein binding assay

GST-NICD was expressed in bacteria and purified as described in Guo *et al.*, 1996. ^{35}S-labelled Numb-N (aa 1-224), Numb-C (aa 224-547) and full length Nle were synthesised by in vitro transcription/translation using the TNT system (Promega). Binding reactions were carried out with 10µl of labeled protein and 5µl of GST or GST-NICD coupled beads in 400µl of PBS 0.1% NP-40 for 1 hour at room temperature. The beads were washed 6 times in PBS, proteins eluted in SDS-gel sample buffer, separated on 10% SDS-polyacrylamide gels and visualised by autoradiography.

Immunoprecipitation

Schneider S2 cells were grown at 25°C in Schneider's medium (Gibco-BRL) with 1% fetal calf serum and 1% Gentamicin. Cells were harvested and transferred into 6-well 30 mm diameter tissue culture plates at 75% confluence. Each well was then rinsed 3 times with Schneider medium without serum and incubated with 10 µg of DNA in 500 µl of Schneider medium and 50 µl of Lipofectin (Gibco-BRL) for 6 hours. Cells were incubated overnight in medium without Lipofectin. Expression was induced by adding $CuSO_4$ to 0.7 mM and incubating for 12 hours. Cells were harvested and lysed by sonication in PBS, 50 mM NaCl, 5 mM EDTA, 5 mM DTT, 1% Triton X-100 containing protease inhibitors (1 mM PMSF, 5 µg/ml aprotinin and leupeptin). Cells debris was removed by 10,000 xG centrifugation. 500 µl of extract (corresponding to $1x10^6$ cells) was incubated with 3 µl of Rabbit anti-HA antibody for 1 hour at 4°C followed by 1 hour at 4°C with 20 µl of a 50% slurry of Protein A-Sepharose beads (Pharmacia). The beads were washed 4 times with lysis buffer, proteins eluted in SDS-gel sample buffer and run on a 6% SDS-polyacrylamide gel. The gel was electrophoretically transferred to Immobilon-P membrane (Millipore), blocked for 1 hour at room temperature in 5% dry milk in TTBS (10 mM Tris pH 8.0, 150 mM NaCl, 0.2% Tween-20) and incubated overnight at 4°C with mouse-anti Notch (9C6; used at 1:2000) or mouse anti-HA (1:1000). The membrane was washed 3 times 5 min in TTBS and incubated for 1 hour with peroxidase-conjugated goat-anti-mouse IgG (Jackson labs) diluted 1:5000 in TTBS. The blot was washed 3 times for 5 min in TTBS and developed using ECL reagents (Amersham).

Example 1: Genetic characterisation of a novel modifier of *Notch* activity

notchoid ^{1} (nd^{1}) is a viable mutant allele of *Notch* that causes scalloping of the wing (Figure 2C). The severity of the nd^{1} phenotype is sensitive to the level of activity at other loci encoding components of both the Notch and Wingless signalling pathways. Thus nd^{1} provides a sensitised genetic background in which to screen for modifiers of Notch signalling activity.

The BDGP collection of P-element induced lethal mutations (Spradling *et al.*, 1995) was screened for dominant modifiers of the nd^{1} phenotype. Several P-element induced mutants were found to enhance the severity of nd^{1} (not shown). One P-element induced mutant, l(2)k13714, was found that suppresses the scalloping of nd^{1} wings (Figure 2C, D). On the basis of its ability to dominantly suppress scalloping of the wing, the gene identified by the l(2)k13714 P-element was called *Notchless* (*Nle*).

To verify that the gene mutated by the P-element is responsible for the mutant phenotype, strains were generated in which the original P-element had been removed by transposase-mediated excision. These chromosomes differ from the original l(2)k13714 chromosome only by the lack of the P-element and fail to suppress the nd^{1} phenotype (data not shown). Although l(2)k13714 comes from a collection of P-elements that are supposed to be lethal mutations, it was noted that homozygous mutant individuals are recovered in this stock. They are morphologically normal, though males are sterile (not shown).

The scalloping of nd^{1} mutant wings is thought to be caused by reduced Wingless activity because overexpression of Wingless can suppress the phenotype (Couso and Martinez Arias, 1994) and because further reducing *wingless* activity enhances the nd^{1} phenotype (Hing *et al.*, 1994). Removing one copy of the *Su(H)* gene enhances the severity of the nd^{1} phenotype and causes an obvious reduction of Wingless expression at the DV boundary (relative to the level in wild-type, compare Figure 2B and E; nd^{1} *Su(H)/+*). Wingless is restored to wild-type levels and the loss of wing tissue is completely suppressed when the *Notchless* mutant is introduced in this background (Figure 2F; nd^{1} *Su(H)/Nle*). *Notchless* also suppresses the phenotypes of nd^{fa} (Figure

2G, H) and nd^2 (data not shown), indicating that the genetic interaction is not specific to one particular allele of *Notch*.

The scalloping phenotype of *nd* alleles is thought to be due to reduced Notch function. Notch signalling through Su(H) is required to induce Wingless at the wing margin (Diaz-Benjumea and Cohen, 1995). Reducing *Su(H)* gene dosage enhances the nd^1 phenotype. Introducing one copy of the *Notchless* mutant was found to restore Wingless expression in the nd^1 *Su(H)/+* background. This suggested that reducing Notchless activity increases Notch activity at the DV boundary of the wing disc.

Example 2: Cloning the Notchless gene

The P element insertion in l(2)k13714 was mapped to cytological position 21C7-8 by the BDGP (Flybase), between the breakpoints of two large deletions Df(2L)al and Df(2L)ast^1 (Figure 3A). Neither of these deletions acts as a dominant suppressor of nd^1 (data not shown), suggesting that the *Notchless* gene lies in the interval between them.

DNA flanking the P element was cloned by plasmid rescue using *Eco*RI digested genomic DNA. A 2.5Kb *Eco*RI-*Hind*III fragment (devoid of P-element sequences) was used to screen a chromosomal walk kindly provided by Markus Noll (Institut fur Molekularbiologie II der Universitat Zurich, Switzerland). The rescue fragment hybridised to a 3.3Kb *Eco*RI fragment. Sequencing of the 3.3Kb DNA fragment revealed the presence of open reading frames on both sides of the P-element insert but in opposite orientation. Genomic rescue suggested that the gene was encoded by the 1.5Kb transcript (to the right of the insert in Figure 3). A 1.1Kb *Eco*RI-*Cla*I fragment to the right of the insertion site containing part of the predicted transcription unit was used to screen a λgt10 eye disc cDNA library (kindly provided by G. Rubin; University of California, Berkeley CA). 6 cDNA clones were isolated. One encodes a putative full-length *Nle* cDNA of 1.5Kb. The rescued DNA hybridised to a 3.3Kb *Eco*RI fragment of λ phage Y2-6. Sequence of the genomic flank identified transcription units on both sides of the P-element insertion (Figure 3A).

The 1.5Kb transcript was identified as the *Notchless* gene by two criteria: (i) the 15Kb SalI fragment of phage Y2-6 was able to restore *Notchless* activity when introduced into

a *nd*^{1} *Nle/+* mutant background (data not shown). The transgene contains all of the 1.5Kb transcription unit but only part of the other transcription unit. (ii) Expression of the 1.5Kb cDNA under GAL4 control restores full *Nle* activity. *nd* ^{1}; *Nle/+* mutant flies carrying a GAL4 driver show the suppressed *nd*^{1} phenotype (Figure 3B). The wing notching phenotype was restored when the 1.5Kb transcript is expressed in the wing disc under GAL4 control in the *nd*^{1}; *Nle/+* mutant (compare Figure 3B and C). Thus increasing the amount of *Notchless* product using GAL4 counteracts the effects of the *Nle* mutant and alleviates the suppression of the *nd*^{1} mutant phenotype. This indicates that the *Nle* mutant phenotype is due to reduced gene activity.

The P-element insertion that causes the mutation is located 310 bp 5' to the start of the *Nle* open reading frame. It is therefore likely that the P-element mutant reduces the level of *Nle* expression. To obtain a deletion that removes the *Nle* locus, mutants were generated by mobilisation of the P-element. An excision mutant named *Nle*$^{\Delta 8}$ deletes sequences on both sides of the insertion (Figure 3A).

To ask whether the *Nle*$^{\Delta 8}$ deletion allele would produce a stronger increase in Notch activity than the l(2)k13714 P-element insertion mutant, suppression of the *nd*^{1} phenotype were first examined. We observed no difference in the extent of suppression of *nd*^{1} (data not shown). The *Nle*$^{\Delta 8}$ deletion is embryonic lethal when homozygous, but deletes at least one additional transcription unit. Bearing in mind that any phenotypes produced by the deletion could be attributed to its being mutant for more than one gene, homozygous *Nle*$^{\Delta 8}$ embryos and clones of *Nle*$^{\Delta 8}$ mutant cells were examined for neurogenic phenotypes. No difference was detected between mutant and wild-type embryos in the developing PNS and CNS, visualised by 22C10 antibody (data not shown). Likewise, we did not observe any bristle pattern abnormality in the notum or wing of homozygous *Nle*$^{\Delta 8}$ mutant clones (data not shown).

Flies heterozygous for the P-element insertion and the *Nle*$^{\Delta 8}$ deletion are viable, morphologically normal and male sterile, like the homozygous P-element mutant. Together these observations suggest that the original P-element mutant may be a null

allele of *Nle*. The lethality caused by the $Nle^{\Delta 8}$ deletion is likely to be due to another gene.

Example 3: *Notchless* enhances the effects of mutants that increase Notch activity

Certain *Abruptex* alleles of *Notch* have been classified as mutations that increase Notch activity. Their phenotypes are enhanced by increasing the level of wild-type *Notch* gene product and are suppressed by reducing it (de Celis and Garcia-Bellido, 1994; Brennan *et al.*, 1997). Like other gain-of-function *Abruptex* alleles Ax^{28} flies show reduced numbers of some bristles on the head and thorax, as well as shortening of wing veins (Figure 4A, B). These phenotypes are made more severe by introducing an extra copy of the wild-type *Notch* gene (data not shown). They are also enhanced by removing one copy of the *Notchless* gene (Figure 4C). The shortening of the wing veins is more pronounced in Ax^{28} *Nle*/+ flies (arrows). Ax^{28} *Nle*/+ flies show increased loss of both small bristles in the thorax (note the large bare patch outlined in red, Figure 4C), and of large bristles in the head compared with Ax^{28} flies. Blue shading on the head indicates the cluster of orbital bristles. There are three in wild-type flies, one or two in Ax^{28} flies and none in Ax^{28} *Nle*/+ flies. Thus removing one copy of *Nle* enhances the severity of the phenotypes caused by increased Notch activity in Ax^{28} flies.

It was observed that wing veins are reduced in mutant combinations involving nd^1 and *Nle*/+ (Figure 2D, F). Similar results were obtained with nd^2 (data not shown). This phenotype is likely to reflect increased Notch activity. Matsuno *et al.*, (1995) have observed loss of wing veins in nd^1 heterozygous flies (which are themselves morphologically normal) when a low level of the activated form of Notch is expressed under heat-shock control. Together these observations suggest that the nd^1 mutation shows an abnormal increase in Notch activity in wing vein formation. By analogy to the effects of expressing the activated form of Notch (Matsuno *et al.*, 1995) it is probable that the effect of the *Nle* mutation is to further increase the aberrant Notch activity in the nd^1 mutation. These results appear to be at odds with the observation that the nd^1 mutation reduces Notch function at the wing margin (Figure 2). This suggests that the

nd^{1} mutation behaves as a loss of function allele in one context and as a gain of function allele in another.

Example 4: *Notchless* opposes *deltex* function

Deltex is thought to function as a positive regulator of Notch activity (Diederich *et al.*, 1994; Matsuno *et al.*, 1995). *deltex* mutant flies show a phenotype resembling a reduction of Notch activity: nicking of the distal region of the wing blade and thickening of the wing veins (Figure 5A). Removing one copy of *Notchless* restores the *deltex* mutant wing to normal (Figure 5B). Thus the effects of reducing *deltex* activity can be compensated for by simultaneously reducing *Notchless* activity. Likewise, removing one copy of *Notchless* enhances the effects of overexpressing Deltex using a heat shock-*deltex* transgene (Matsuno *et al.*, 1995). Under conditions where Deltex overexpression produces no visible abnormality in an otherwise wild-type wing (Figure 5C), it causes loss of veins in a *Nle*/+ background (arrow, Figure 5D). This resembles the effects of increasing Notch activity in *Abruptex* mutants.

These results suggest that Deltex and Notchless act in opposite directions as modifiers of Notch activity in wing development.

Example 5: Notchless expression in Xenopus

XNle was isolated by PCR using the degenerate primers, F 5'-CGC AGA ATT CCI TTY GAY GTI CCI GTI GAY AT-3' and R 5'-GGT GCT CGA GCY TGI GGY TGR TAI ATD ATR TC-3', designed against conserved peptides, PFDVPVDI and DIIYQPQ respectively, found in the Nle domain of the vertebrate proteins identified as expressed sequence tags.

Phage stock of a stage 30 library (Stratagene) was used as template to amplify a 200 bp fragment that spans the Nle domain. Five independent clones were sequenced and found to be identical. This fragment was used to screen the stage 30 library, which resulted in the isolation of 25 positive clones of which the longest of 2.2Kb was sequenced on both strands. Temporal expression was assayed by RT-PCR analysis as described by Bouwmeester *et al.*, 1996 using the following primer set that amplifies a XNle fragment

of 135 bp; F 5'-CAC CAG ATA AAC TGC AGT TAG-3', R 5'-CTG TTT CAA CTG ATT GCT TCT-3' (28 cycles).

Spatial expression was analysed by whole-mount in situ hybridisation essentially as described (Bouwmeester *et al.*, 1996), using antisense RNA synthesised from pBS-*XNle* linearised with *Xho*I and transcribed with T3 polymerase.

For injection purposes pCS2-*XNle* was constructed by subcloning of a 2.2Kb *Eco*RI fragment in the complementary site of pCS2+. Capped RNA was synthesised using pCS2-*XNle*, pCS2-*Drosophila Nle* (kindly provided by J. Wittbrodt; Max-Planck Institut fur Biophysikalischechemie, Gottingen, Germany) and pCS2-*NOTCHI-ICD* (kindly provided by C. Kintner; Salk Institute, San Diego CA) digested with Not-1 and transcribed with Sp6. Synthetic RNA (2.5 - 5 ng of *XNle* and *DNle* RNA, 100-200 pg *XN-ICD*) was injected into one blastomere of the 2-cell stage embryo. Embryos were harvested at early neurula stage (st. 13-15). β-galactosidase activity, a lineage marker for injections, was revealed using X-gal as substrate prior to whole mount in situ. Primary neurons were identified by β-tubulin staining. Antisense β-tubulin RNA was synthesised from pBS- β-tubulin digested with Not I and transcribed with T3 polymerase.

Figure 7 shows the temporal expression of *XNle*. Total RNA isolated from the indicated stages of development was analyzed by RT-PCR analysis for expression of *XNle* and *Histone H4* (loading control). E, egg; 4C, 4 cell stage; all other lanes are labelled with stage numbers according to Nieuwkoop and Faber (1956) Normal table of Xenopus Laevis A. Systematical and chronologicla survey of the development from the fertilised egg until the end of metamorphosis. *North Holland publishing company, Amsterdam.* (B) Spatial expression of *XNle*. Whole mount in situ hybridization was used to visualize expression of XNle at neural plate stage (st. 17), tailbud stage (st. 25) and tadpole stage (st. 35). Expression patterns are described in the text. symbols: NC, neural crest; pm, paraxial mesoderm; b, brain; e, eye; ba, branchial arches; s, somites; sp, segmental plate; vbi, ventral blood islands. (C) Phenotypic consequence of overexpression of *XNle*, *DNle* and an activated form of *Xenopus NotchI* (XN-ICD) on primary neurogenesis. LacZ RNA was co-injected to mark the injected side. Control embryo: l, i, m denote lateral, intermediate and medial rows of β-tubulin expressing primary

neurons. Note the reduction in the number of primary neurons on the injected side in embryos injected with *XNle*, *DNle* or *N-ICD*. Arrows indicate the absence of lateral and intermediate neurons in XNle and DNle injected embryos and all neurons in *XN-ICD*-injected embryo. In B and C anterior is to the left.

This figure shows that the *Xenopus Notchless* gene (*XNle*) is maternally transcribed and that expression remains relatively constant during the early stages of embryonic development without obvious signs of localisation. Elevated levels arise at the end of gastrulation and are maintained during neurulation and organogenesis (Figure 7A). Localised expression is observed in two lateral domains adjacent to the rostral neural plate, which correspond to the premigratory neural crest cells, and in a region at the anterior end of the neural plate, which corresponds to placodal precursors (Figure 7B). There is also increased expression in the involuting paraxial mesoderm and in two patches lateral to the closing slit blastopore, through which future somitic cells involute.

During subsequent stages expression is evident in the somites and unsegmented paraxial mesoderm, the segmental plate. High levels are also seen in the head region; in the branchial arches, eyes and different regions of the developing brain (Figure 7B, st.25). Later on expression is in addition found in two patches on the ventral site of the embryo, the ventral blood islands which generate the hematopoietic precursors of the early embryo (Figure 7B, st.35).

The pattern of *XNle* expression resembles that of other components of the Notch pathway, including *Delta* and *Kuzbanian* (Chitnis *et al.*, 1995; Pan and Rubin, 1997). These expression domains correspond to regions where Notch signalling has been implicated in cell fate specification events (Chitnis *et al.*, 1995; Coffman *et al.*, 1993; Jen *et al.*, 1997).

Example 6: Overexpressing Notchless increases Notch activity

Based on the finding that reducing Nle activity increases Notch activity in *Drosophila* (Figs 1-4), it was anticipated that overexpression of Nle would reduce Notch activity. To test this proposal the *Xenopus* neuronal specification assay was used (Chitnis *et al.*, 1995). Notch signalling is involved in controlling the choice between neural and epidermal fate. Overexpression of activated forms of Notch reduces the number of cells

adopting neural fate in *Xenopus* (Chitnis *et al.*, 1995). Conversely, reduction of Notch activity would be expected to increase the number of cells adopting neural fate, as in *Notch* mutant embryos in *Drosophila* (Campos-Ortega and Jan, 1991). Surprisingly, it was observed that overexpression of XNle and of *Drosophila* Nle reduces the number of neurons, as in the activated Notch control (Figure 7C). Although high levels of *Nle* RNA were injected, no sign of other developmental defects was observed: gastrulation and subsequent morphogenesis proceeds normally.

This unexpected finding led us to test whether overexpression of Nle in *Drosophila* would have a comparable effect on neural fate specification. Expression of activated Notch reduces thoracic bristle formation (Struhl *et al.*, 1993; Rebay *et al.*, 1993). *UAS-Nle* was expressed in the notum under control of apterous-GAL4. The number of small bristles was found to be reduced in flies expressing *UAS-Nle* compared to *apterous-GAL4* alone (Table 1). Although the reduction is not large in magnitude, it is statistically significant ($p<0.0001$). Thus overexpression of Nle increases Notch activity in both *Xenopus* and *Drosophila* neural fate specification.

Table 1: Suppression of neural fate by overexpression of Notchless

	# microcheate/notum	
genotype	mean ± SE (n)	t-test
ap-Gal4/+	214 ± 2 (17)	
ap-Gal4/UAS-Nle	196 ± 3 (17)	$p< 0.00001$

Wild-type flies have on average 260 small bristles per thorax (Brennan *et al.*, 1997). This number is reduced in Apterous-GAL4/+ flies. Expression of Nle further reduces the number of bristles.

Example 7: Notchless protein binds to the intracellular domain of Notch

To ask whether Nle might regulate Notch through direct protein interaction we carried out GST pull down and immunoprecipitation assays. *In vitro* binding was tested using ^{35}S-Met-labeled test proteins and the intracellular domain of Notch expressed in bacteria as a GST-fusion protein (Guo *et al.*, 1996). Figure 8A shows binding of Nle, Numb-N and Numb-C to GST-Notch-ICD and GST control proteins. N-terminal and C-terminal fragments of Numb were used as controls for binding to the intracellular domain of Notch (Guo *et al.*, 1996). Input lanes show 1/10 of the input to the binding reaction. GST-N indicates GST-Notch-ICD beads; GST indicates GST-beads. Numb-N and Nle (arrow) bind to GST-N beads more strongly than to GST control beads. Coomassie blue staining of the gel (not shown) showed that there was significantly more protein bound to the GST control beads than to the GST-N beads.

To conclude, Figure 8A shows weak non-specific binding of GST control beads to all three proteins, but this is well below the level of specific binding observed with Numb-N and Notchless.

In vivo interaction between Notchless and Notch in *Drosophila* S2 cells was tested by immunoprecipitation. Expression of full-length Notch and HA-tagged Notchless proteins was monitored by immunoblotting of total cell extracts (see below). Extracts from induced and uninduced cells were immunoprecipitated using antibody to the HA-tag, and a blot of the gel was probed with a monoclonal antibody directed against the intracellular part of Notch and reprobed subsequently with anti-HA to visualise the immunoprecipitated HA-Notchless.

Figure 8B shows the results of the immunoprecipitation of Notch and Nle expressed in S2 cells. Upper panel: blot probed with mouse monoclonal anti-Notch (9C6). Lower panel, same blot probed subsequently with mouse anti-HA. Lanes 1-3: total cell lysates from S2 cells expressing (1) Notch (2) HA-Nle or (3) both proteins. Note that low levels of endogenous Notch are seen in the Nle-expressing cells (lane 2). Lanes 4-7: Immunoprecipitates from cells expressing (4)Notch, (5) HA- Notchless or (6, 7) both proteins. + indicates immunoprecipitated with Rabbit anti-HA and protein A beads. - indicates control precipitation with protein A beads alone. Immunoprecipitation of HA-Nle coprecipitates Notch in cells that overexpress both proteins (lane 7). Neither protein

is recovered in control precipitations lacking the anti-HA (lane 6) or in which HA-Nle was not expressed (lane 4). Immunoprecipitation in cells transfected with HA-Nle alone did not detectably coprecipitate Notch (lane 5), although endogenous Notch can be detected in cells not transfected with the inducible Notch expression construct (lane 2). Recovery of HA-Nle was lower in the reaction in lane 5 than in lane 7.

Notch protein was thus found to immunoprecipitate with HA-Notchless from cells expressing both proteins (Figure 8B lane 7). No precipitation was observed in controls lacking HA-Nle or anti-HA (lanes 4, 6). Together these results indicate that Notchless binds directly to the intracellular domain of Notch.

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